RESEARCH

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Discovery

Isolation, production and characterization of protease from *Bacillus subtilis* isolated from soil

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ABSTRACT

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers of these, strains, *Bacillus sp*. dominate the industrial sector. Gram-positive, spore- forming bacterium *Bacillus subtilis* produces and secretes proteases, esterases, and other kinds of exo-enzymes at the end of the exponential phase of growth. Proteolytic enzymes from microorganisms may be located within the cell (intracellular), cell wall associated (periplasmic), or excreted into the media (extracelluar). The physiological function of proteases is necessary for all living organisms, from viruses to humans, and proteolytic enzymes can be classified based on their origin: microbial (bacterial, fungal and viral), plant, animal and human enzymes. Action of the proteolytic enzymes include digestion of food proteins, protein turnover, cell division, blood-clotting cascade, signal transduction, processing of polypeptide hormones, apoptosis and the life-cycle of several disease-causing organisms including the replication of retroviruses . The present study is an attempt to validate its significant role in Biological Research and paves way for greater usage in Medical field in the near future.

Keywords: Proteolytic enzymes, Bacillus subtilis, Physiological function, Key role and Medical Field.

1. INTRODUCTION

Extracellular enzymes are used in the food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis. Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale. The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Ammar et al., 2000). Members of the *Bacillus* genus are generally found in soil and most of these bacteria have the ability to disintegrate proteins, namely proteolytic activity (Gupta et al., 2002). Protease enzymes not only have important industrial uses, but also the proteases of these microorganisms play an important role in the nitrogen cycle, which contributes to the fertility of the soil. In paddy field soil, most of the nitrogen source is stored as biomass protein and decomposes slowly to low molecular weight amino acids by the activity of soil protease. Soil protease is thought to be mainly supplied by soil microorganisms (Banerjee et al., 1999).

2. PROTEASE

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks (Anwar, A. and Saleemuddin, 1997). Examples of proteases include: fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin. Proteolytic enzymes are very important in digestion as they breakdown the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy (Ammar et al., 2000). Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control and immune regulation (Gerze et al., 2005).

Proteases are classified into six groups: Serine proteases, threonine proteases, cistern proteases, aspartate proteases, metallo proteases and glutamic acid proteases. Serine proteases are enzymes that cleave peptide bonds in proteins, in which the amino acid is at the (enzyme's) active site. They are found ubiquitously in both eukaryotes and prokaryotes. Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) and orsubtilisin-like (Yang SS and Lee CM, 2001). In humans, they are responsible for co-ordinating various physiological functions, including digestion, immune response, blood coagulation and reproduction. Cysteine proteases also known as thiol proteases, are enzymes that degrade proteins. These proteases share a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad or dyad. Cysteine proteases are commonly encountered in fruits including the papaya, pineapple, fig and kiwifruit (Kumar et al., 1999). The proportion of protease tends to be higher when the fruit is unripe. In fact, dozens of lattices of different plant families are known to contain cysteine proteases. Cysteine proteases are used as an ingredient in meat tenderizers. Threnonine proteases are a family of proteolytic enzymes harbouring a threonine (Thr) residue within the active site (Paliwal et al., 1994). The prototype members of this class of enzymes are the catalytic subunits of the proteasome, however the acyltransferases convergently evolved the same active site geometry and mechanism (Sandhya et al., 2004). A metalloproteinase, or metalloprotease, is any protease enzyme whose catalytic mechanism involves a metal. An example of this would be meltrin which plays a significant role in the fusion of muscle cells during embryo development, in a process known asmyogenesis. Most metalloproteases require zinc, but some use cobalt. The metal ion is coordinated to the protein via three ligands (Kumar et al., 2002). The ligands co-ordinating the metal ion can vary with histidine, glutamate, aspartate, lysine, and arginine. The fourth coordination position is taken up by a labile water molecule. Treatment with chelating agents such as EDTA leads to complete inactivation. EDTA is a metal chelator that removes zinc, which is essential for activity (Schickaneder E. 1988). They are also inhibited by the chelator orthophenanthroline. Aspartic proteases are a family of protease enzymes that use an aspartate residue for catalysis of their peptide substrates. In general, they have two highly conserved aspartates in the active site and are optimally active at acidic pH. Nearly all known aspartyl proteases are inhibited by pepstatin (Gerze et al., 2005).

3. MATERIALS AND METHODS

Collection and preparation of soil samples

In systematic screening program for isolation of bacteria, soil samples were collected in Women's Christian College campus. Soil samples (approximately 5g) were collected using some clean dry and sterile polythene bag along with spatula. 1g of soil samples were dissolved in 10ml of water to make soil suspensions.

Isolation of bacteria

The media used in this research was nutrient agar medium.

Peptone - 0.3g
Agar - 2g
Yeast extract - 0.69g
NaCl - 1.75g
Nutrient agar medium - 0.5%

It was stirred vigorously and dissolved using hot plate after which it was sterilized in autoclave for 15mins at 121°C. It was then allowed to cool after which it was dispensed in Petri dishes and allowed to solidify.

Optimal conditions for protease production by *Bacillus subtilis*

- Optimum substrate concentrations - 0.5 %
- Optimum incubation period - 30 hrs
- Optimum incubation temperature - 40 ºC
- Optimum pH - 7.0
- Best buffer for production of protease enzyme - Phosphate buffer
- Optimum inoculum size - 1 ml-1 from stock suspension of *Bacillus subtilis* (7 ×103/ ml-1)
- Optimum inoculum age - 24 hr.
- Optimum fermentor (flask) capacity(aeration) – 250 ml
- The best-extracted volume - 150 ml.
- The best broth ingredient - beef extract and NaCl
- An optimum carbon source - lactose
- Optimum nitrogen source for protease production - (NH4) 2 SO4
- The best amino acids used for the production of protease enzyme which utilized organic acids, acetic, citric, lactic acid decreased protease production at different concentrations – Valine.
- Protease enzyme purified by Ammonium Sulfate Precipitation and sephadex G 200 filtration.

Sample inoculation

Portions of the suspensions were inoculated on milk agar by streaking and were incubated on 37°C for 24 hours after which colonies with a clear zone of inhibition were observed.

Gram's staining

Colonies that was grow on milk agar were stained in accordance with standard gram staining procedure. The slide was heat fixed with the specimen by passing it over a heat source, such as a flame, several times using a forceps. The slide was passed very quickly through the flame so that it is not heated excessively. The slide was placed slide on the staining tray. The fixed smear was flooded with crystal violet solution (#1) and allows to remain for 1minute. The crystal violet was rinsed off with distilled or tap water. The slide was flooded with iodine solution ans was allowed to remain for one minute. The iodine solution was rinsed off with distilled or tap water. The slide was flooded with decolorizer for one to five seconds. The decolorizer was then rinsed off with distilled or tap water. The slide was then flooded with safranin and was allowed to remain for 30 seconds. The safranin was then rinsed off with distilled or tap water. The slide was dried on bibulous paper or absorbent paper and was placed in an upright position.

Biochemical test

The following Biochemical Tests were carried out using standardized protocols

1. INDOLE TEST - to determine whether bacteria can breakdown the amino acid tryptophan into indole.
2. CATALASE TEST - an enzyme that breaks down hydrogen peroxide into water and oxygen. A catalase is a way to grid of the peroxide in a cell.
3. CITRATE UTILIZATION TEST - test which detects the ability of an organism to use citrate (a derivate of citric acid, that is the salts, esters and the polyatomic found in the solution) as the sole source of carbon and energy.
4. MR-VP TEST - Methyl Red and Voges – Prokauer test are among the two various tests used in the biochemical identification of bacterial species. These tests were originally studied by Voges, Proskauer (1) and subsequently by Clark and Lubs (2). This test differentiates between members of the coli- aerogens group. Broth the tests are based on the detection of specific breakdown products of carbohydrate metabolism.
5. OXIDASE TEST - test used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizers the reagent to purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.
6. TRIPLE SUGAR IRON AGAR TEST - Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and pH indicator phenol red. It is used to differentiate enteric based on the ability to reduce sulfer and ferment carbohydrates.

Production of protease from *bacillus sp*

Production of protease from *Bacillus Sp* was carried out in a medium containing the following

- Glucose - 0.5% (wt/vol)
- Peptone - 0.75% (wt/vol)
- Salt solution - 5% (vol/vol)
- (MgSO4.7H2O - 0.5% [wt/vol]; KH2PO4 - 0.5% [wt/vol]

- FeSO4.7H2O - 0.01% [wt/vol]),
- pH - 7.0

It was maintained at 37 °C for 24 hours in a shaker incubator (200 rpm). The pH of the medium was adjusted with 1N NaOH or 1N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.

Init %		20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0		69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5		66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1		62.7
15	2.6	5.4	8.2	11.2	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7		59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2		55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8		52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5		48.8
35				0	2.9	5.7	8.7	11.8	15.1	18.4	21.8	25.8	29.6	32.9	36.9	41.0		45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	26.3	29.6	33.5	37.6		41.8
45						0	3.0	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2		38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.5	26.8	30.8		34.8
55								0	3.1	6.1	9.3	12.7	16.1	20.1	23.5	27.3		31.2
60									0	3.1	6.2	9.5	12.9	16.8	20.1	23.9		27.9
65										0	3.2	6.3	9.7	13.2	16.8	20.5		24.4
70											0	3.2	6.5	9.9	13.4	17.1		20.9
75												0	3.3	6.6	10.1	13.7		17.4
80													0	3.4	6.7	10.3		13.9
85														0	3.4	6.8		10.5
90															0	3.4		7.0
95																0		3.5
100																		0

Partial purification of enzyme by ammonium sulphate precipitation

When the salt concentration is increased, some of the water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the charged part of the protein. As a result of the increased demand for solvent molecules, the protein- protein interactions are stronger than the solvent- solute interactions; the protein molecules coagulate by forming hydrophobic interactions with each other. This method is ammonium sulphate and because of its easy solubility lake of toxicity to most enzymes, low cost and its stabilizing effect on enzymes is used widespread to partially purify the enzyme.

Ammonium sulphate saturation calculation

Dialysis

Diffusion is the random, thermal movement of molecules in solution that leads to the net movement of molecules from an area of higher concentration to a lower concentration until equilibrium is reached. In dialysis, a sample and a buffer solution are separated by a semi permeable membrane that causes differential diffusion patterns, thereby permitting the separation of molecules in both the sample and dialysate.

Analytical methods for determination of protease activity

1ml of the substrate buffer was taken into different clean test tubes. 2ml of the enzyme was added to the solution and it was incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 2ml of TCA.

The reaction mixture was allowed to precipitate. Then the precipitate was centrifuged at 10000 rpm for 20 minutes. 0.5 ml of supernatant was drawn from each test tube and make up to 1 ml by the addition of the Tris buffer. 2ml of Sodium Hydroxide solution was added to each of the test tubes containing supernatant. The contents were mixed well. 0.5 ml of Folin Ciocalteau reagent was added and incubated at room temperature for 20 minutes. The absorbance was measured at 620 nm after the development of blue colour using UV-Visible spectrophotometer. A control was also performed by adding the enzyme after the inactivation of the substrate by the addition of TCA. The tyrosine released by the enzyme was calculated from tyrosine standard graph.

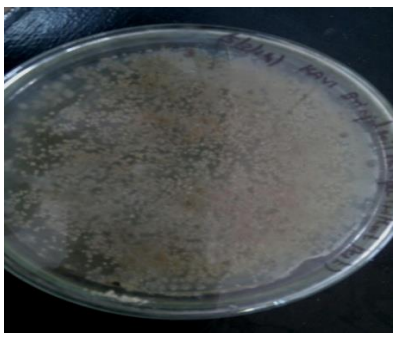


Figure 1
Bacillus subtilis

Figure 2
Indole test

Figure 3
Citrate Test

Figure 4
MR-VP

Figure 5
Catalase Test

Figure 6
Oxidase Test

Figure 7
Milk Agar Plate

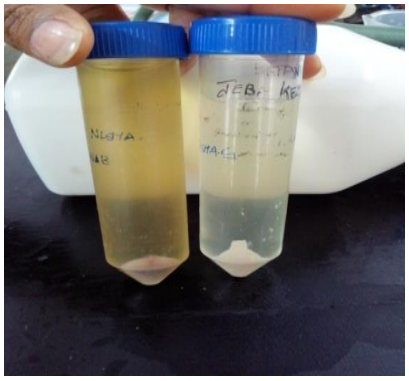


Figure 8
Centrifugation

Figure 9
Dialysis

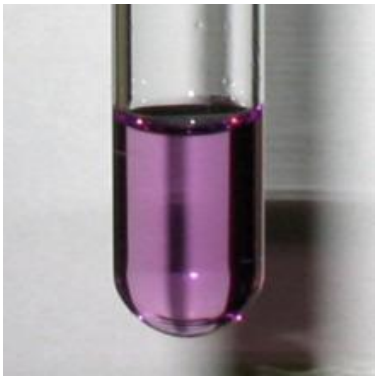


Figure 10
Protein estimation by Lowry method

Estimation of protein by lowry's method

Protein was estimated using Lowry's method (1951).

Effect of temperature on protease

Four sets of test tube were taken and marked as T_1,T_2,T_3,T_4 and C_1,C_2,C_3,C_4. 1ml of phosphate buffer was added to all test tubes.1ml of substrate solution was added to all the test tubes. 1ml of manganese sulphate activator was added to all the test tubes. A tube containing distilled water is maintained as blank. The tubes were pre-incubated at room temperature for 15 minutes.0.5ml of enzyme extract was added to the test tubes T_1 –T_4. The test tubes were incubated at various temperature for about 15 minute .The activity of the enzyme was arrested by adding 2ml of 1% Sodium hydroxide. 0.5ml of enzyme extract was added to the test tube C_1 – C_4. The tubes were mixed and then incubated in boiling water bath for 10 minutes. 2ml of ninhydrin solution was added to all the test tubes. The tubes were incubated in a boiling water bath for 15 minutes. 3ml of 30% ethanol was added to all test tubes and shaken well. The intensity of the purplish blue colour developed was read at 570nm. The graph was plotted with optical density against temperature and the optimum temperature was determined.

Effect of pH on protease

Four sets of test tube were taken and marked as T_1,T_2,T_3,T_4 and C_1,C_2,C_3,C_4. 1ml of phosphate buffer of varying pH was added to all test tubes. 1ml of substrate solution was added to all the test tubes.1ml of manganese sulphate activator was added to all the test tubes. A tube containing distilled water is maintained as blank. The tubes were preincubated at room temperature for 15 minutes. 0.5ml of enzyme extract was added to the test tubes T_1 –T_4. The test tubes were incubated at room temperature for about 10 minutes. The activity of the enzyme was arrested by adding 2ml of 1% Sodium hydroxide. 0.5ml of enzyme extract was added to the test tube C_1 –C_4. The tubes were mixed and then incubated in boiling water bath for 10 minutes. 2ml of ninhydrin solution was added to all the test tubes. The tubes were incubated in a boiling water bath for 15 minutes. 3ml of 30% ethanol was added to all test tubes and shaken well.The intensity of the purplish blue colour developed was read at 570nm.The graph was plotted with optical density against pH and the optimum temperature was determined.

Enzyme immobilization by gel entrapment

An Immobilized enzyme is an enzyme that is attached to inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can be provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again a far more efficient process and so is widely used in industry for enzyme catalysed reactions. An alternative to enzyme immobilization is whole cell immobilization. 4% $CaCl_2$ was mixed in 100ml water and kept for 2 hours incubation at 4 ˚C. 3.5gm and 0.685gm of sodium alginate and sodium alginate was dissolved in 100 ml of distilled water respectively and was left undisturbed for 30 minutes to eliminate the air bubbles. 2 - 4 ml of enzyme was approximately mixed with 10 ml sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 4%$CaCl_2$ solution with a syringe and a needle at room temperature.

4. RESULTS AND DISCUSSION

Isolation of *bacillus subtilis* from soil

Soil was collected near the college campus. *Bacillus subtilis* was isolated from the soil sample. It was cream colour (on nutrient agar), flat and circular with undulate margin (Figure 1).

Scientific classification

Domain : Bacteria
Phylum : Firmicutes
Class : Bacilli
Order : Bacillales
Family : Bacillaceae
Genus : Bacillus
Species : B. subtilis

Biochemical test for *bacillus subtilis*

The results were obtained through Biochemical Tests (Table 1 & Figure 2 – 6)

Table 1

Bichemical Tests

TESTS	ORGANISM	COLOR	RESULT
INDOLE TEST	*Bacillus sp*	NO PINK RING	NEGATIVE
CATALASE TEST	*Bacillus sp*	BUBBLES WERE OBSERVED	POSITIVE
CITRATE TEST	*Bacillus sp*	BLUE	POSITIVE
METHYL RED TEST	*Bacillus sp*	NO BROWN COLOUR	NEGATIVE
VOGES PROSKAUER TEST	*Bacillus sp*	RED	POSITIVE
OXIDASE TEST	*Bacillus sp*	DARK BLUE	POSITIVE
TRIPLE SUGAR IRON AGAR TEST	*Bacillus sp*	BLACK	POSITIVE

Table 2

Protein estimation by Lowry method

S.NO	REAGENT	B	S1		S2		S3		S4		S5		U
1	VOLUME OF STANDARD	-	0.2		0.4		0.6		0.8		1		-
2	DISTILLED WATER	1	0.8		0.6		0.4		0.2		0		-

3	ALKALINE COPPER SOLUTION	5	5		5		5		5		5		5
		SHAKE WELL AND ALLOW TO STAND FOR 10 MINS											
4	DILUTED FOLIN'S REAGENT	0.5	0.5		0.5		0.5		0.5		0.5		0.5
		INCUBATE AT ROOM TEMPERATURE IN THE DARK FOR 30 MINS											
5	OPTIMAL DENSITY(660nm)	0	0.03		0.06		0.09		0.12		0.15		0.17

Table 3
Effect of temperature on protease

Temperature	Absorbance at 570 nm
30°C	1.39
40°C	1.65
50°C	1.57
60°C	1.45
70°C	1.43

Table 4
Effect of Ph on Protease Enzyme

p H	Absorbance at 570 nm
5	1.03
6	1.14
7	1.6
8	1.41
9	1.51

Production of protease in milk agar plate
After 24 hours incubation plates were observed with zone of clearance indicates the production of extra celluar enzyme. Milk agar plates showing zone of clearance indicates the production of extra cellular protease (Figure 7).

Centrifugation
The whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered (Figure 8).

Ammonium sulphate precipitation
It's a method used to quickly remove large amounts of contaminant proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Each protein precipitate was dissolved individually in fresh buffer and assayed for total protein content and amount of desired protein.

Dialysis
In dialysis, the sample and the buffer solution (called the dialysate) were separated by the semi- permeable that causes differential diffusion patterns, thereby permitting the separation of molecules in the sample dialysate. Thus the sample was purified (Figure 9).

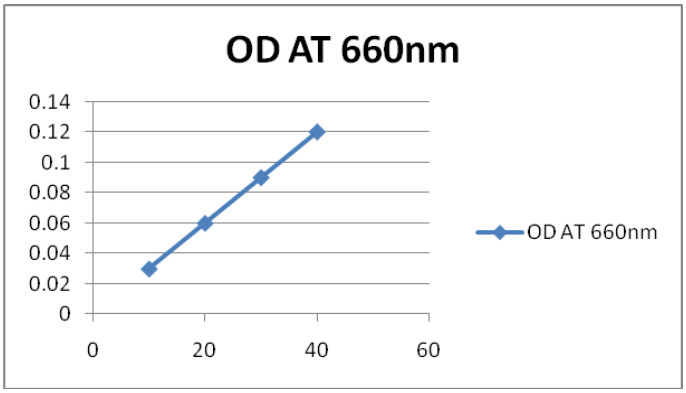
Estimation of protein by lowry's method
Protein was estimated using Lowry Method and the following results were obtained (Figure 10, Table 2 & Graph 1)

Effect of temperature on protease enzyme
The activity of the purified enzyme was determined at different temperatures ranging from 30°C to 70°C. The optimum temperature recorded was at 40°C for protease activity (Figure 11, Table 3 & Graph 2).

Effect of pH on protease enzyme
The highest protease activity was found to be at pH 7-8 (Figure 12, Table 4 & Graph 3).

Protease assay for sample
The value was taken in UV spectrometer at 280 nm. Protein was calculated using the formula,
Protein activity (mg/ml) = Difference in the O.D × Dilution factor

Protein activity (mg/ml) = 1000 × 0.05 = 50 U.
Specific activity U/mg = protein activity / protein concentration in mg = 50 / 126
= 0.39 U.



Graph 1
Protein Estimation by Lowry Method

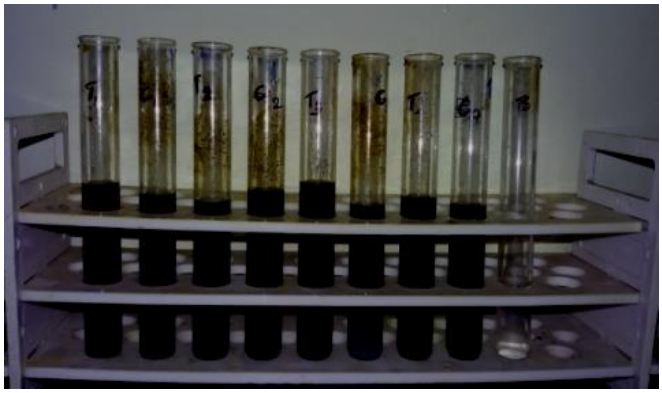
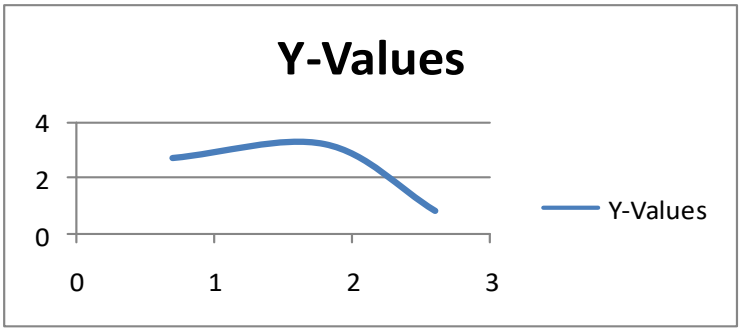


Figure 11
Effect of temperature on protease enzyme



Graph 2
Effect of temperature on protease

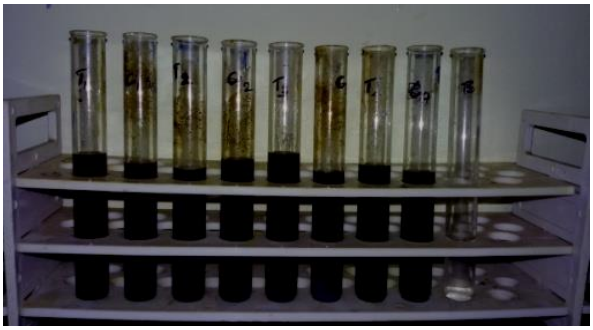
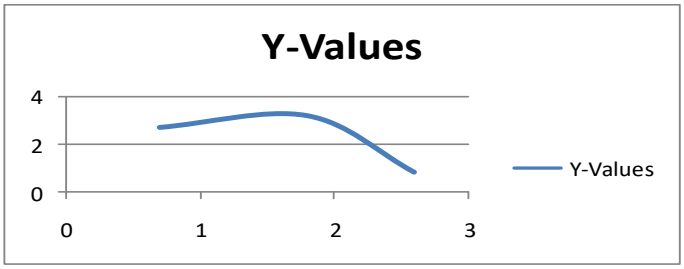


Figure 12
Effect of ph on protease enzyme



Graph 3
Effect of ph on protease enzyme

Figure 13
Gel entrapment

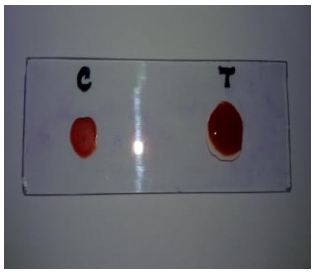


Figure 14
Delayed blood clot

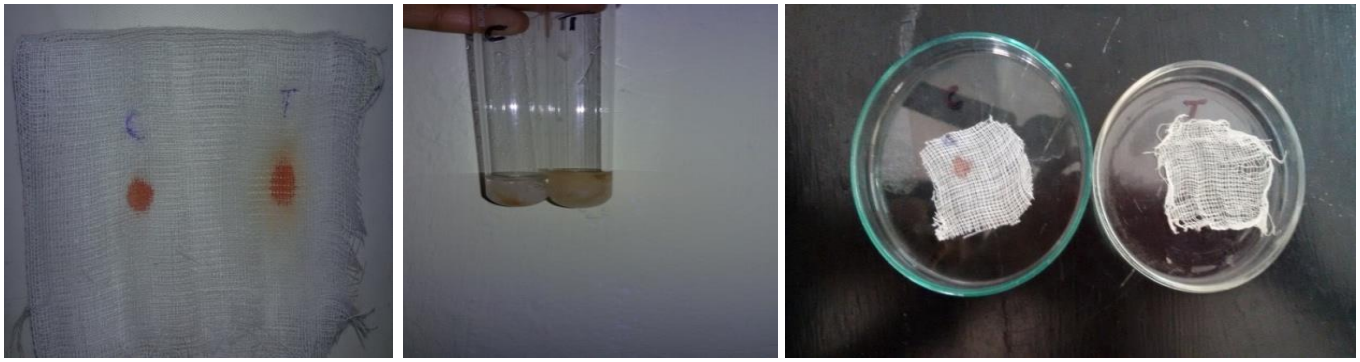


Figure 15
Removal of blood stains

Enzyme immobilization by gel entrapment

There are several reasons for the preparation and use of immobilized enzymes. In addition to a more convenient handling of enzyme preparations the two main targeted benefits are (1) easy separation of the enzyme from the product and (2) reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme application and supports liable and efficient reaction technology. Thus immobilized beads were obtained using immobilization by gel entrapment method (Figure 13). The applications of it being Delayed Blood Clot and removal of Blood Stains (Figures 14 & 15).

5. CONCLUSION

Proteolytic enzyme refers to the various enzymes that digest protein. These enzymes include the pancreatic proteases chymotrypsin and, trypsin, bromelain, papin, fungal proteases, serratia peptidase (the "silk worm" enzyme). Proteolytic enzymes are capable of hydrolyzing peptide bonds and are also referred to as peptidases, proteases or proteinases. The physiological function of proteases is necessary for all living organisms, from viruses to humans. Due to their key role in the life-cycle of many hosts and pathogens they have great medical, pharmaceutical, and academic importance. Thus the present study validates its significant role in Biological Research and paves way for greater usage in Medical field in the near future.

REFERENCE

1. Ammar, MS, El-Louboudy, SS. Abdulraouf, UM. Proteases : properties, sources, applications, and stability *Az. J. Microbiol*. 1991.(13):12-29
2. Anwar A, Saleemuddin M. Chemical stability and properties of proteases.*Biotech. App. Biochem*. 1997. 25: 43-46
3. Anwar A, Saleemuddin M. Isolation of Protease by high speed TLC. *Biotech. App. Biochem*. 2000, 31:85-89
4. Banerjee UC, Sani RK, Azmi W, Soni R. Stability and use of Protease in food industry. Red *Proc. Biochem*., 1999. 35: 213-219
5. Gerze A, Omay D, Guvenilir Y. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus megatherium*. *Appl Biochem Biotechnol.,* 2005 121-124:335-45
6. Kumar CG, Tiwari MP, Jany KD. Biosynthesis, processing, and stability of Protease.*Proc. Biochem*. 1999, 34: 441-449
7. Kumar A, Sachdev A, Balasubramanyam SD, Saxena, AK Lata. Optimization of conditions for production of neutral and alkaline protease from species of bacillus and pseudomonas. *Ind. J. Microbiol.* 2002, 42: 233-236
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RL. Estimation of Protein. *J Biol Chem*. 1951; 193:265-273
9. Sandhya C, Sumantha A, Pandey A. Proteases In: Enzyme Technology, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), Asiatech Publishers Inc., New Delhi, 2004, India pp. 312–325
10. Schickaneder E. Casein-resorufin, a new substrate for a highly senditive protease assay *Fresenius Z. Anal. Chem.* 1998, 330, 360
11. Paliwal N, Singh SP, Garg SK.Cation-induced thermal stability of an alkaline protease from a *Bacillus sp. Bioresource Technol* 1994, 50 : 209 - 11
12. Yang SS and Lee CM. Effect of culture media on protease and oxytetracycline production with mycelium and protoplasts of *Streptomyces rimosus*. *World J. Microbiol Biotech.,* 2001, 17: 403 - 410